Final Report

Mechanisms for Electron Transfer through Electrochemically Active Biofilms

Derek R. Lovley and Kelly P. Nevin
Department of Microbiology
University of Massachusetts
Amherst, MA 01003
Phone: 413-545-9651, Fax: 413-545-1578, Email: dlovley@microbio.umass.edu

Award Number: N000141010084

LONG-TERM GOALS

Microbial fuel cells show promise for powering monitoring devices in remote locations, and the ability of microorganisms to store charge and transfer electrons to electrodes may have applications in bioelectronics. The goal of these studies was to develop a better understanding of the mechanisms for biofilm capacitance and long-range electron transfer through current-producing biofilms to aid in better optimizing these microbial fuel cell and bioelectronic applications.

OBJECTIVES

The objectives of this research were to: 1) evaluate the mechanisms for electron transfer through the anode biofilm of microbial fuel cells and onto the anode surface and 2) determine the components that confer conductivity and capacitance to biofilms in order to evaluate the potential for the use of microbial biofilms in bioelectronics.

APPROACH

These studies primarily focused on strains of *Geobacter sulfurreducens* because: 1) they are closely related to the microorganisms that predominate on the anodes of benthic microbial fuel cells as well as other microbial fuel cells harvesting current under strict anaerobic conditions; 2) they produce the highest current densities of known pure cultures; 3) their biofilms have extraordinary conductance; 4) their genome sequence is available; and 5) they can be manipulated with genetic techniques. Strains developed via adaptive selection or genetic manipulation were evaluated with biophysical and electrochemical approaches to better understand the contribution of different cellular components to the electronic properties of biofilms. Furthermore, (b) (4)

RESULTS

Major discoveries made under this grant included the finding that: 1) the pili of *Geobacter sulfurreducens* are conductive along their length via metallic-like conductivity; 2) *G. sulfurreducens* biofilms are electrically conductive; 3) networks of pili coursing through current-producing biofilms of *G. sulfurreducens* confer metallic-like conductivity to the biofilms; 4) aromatic amino acids in the carboxyl-end of the PilA sequence contribute to the metallic-like conductivity of pili; 5) increasing biofilm conductivity through genetic manipulation or adaptive evolution increases current density in microbial fuel cells; 6) the cytochrome OmcZ is specifically localized at the anode-biofilm interface and is important for facilitating biofilm-to-anode electron transfer; and 7) *G. sulfurreducens* biofilms function as supercapacitors and the high capacitance can be attributed to the abundance of *c*-type cytochromes.

When these studies were initiated there was considerable confusion in the literature about the importance of biofilm conductivity in contributing to the current density of microbial fuel cells. This could be attributed in part to the fact no one had actually directly measured biofilm conductivity. We developed a strategy for making these measurements and demonstrated that the biofilms of *Geobacter sulfurreducens* are highly conductive. Surprisingly, treating the biofilm with a reagent that denatured the cytochromes had no effect on conductance. Electrochemical gating and temperature dependence studies suggested that the high biofilm conductivity could be attributed to metallic-like conductivity, similar to that previously observed in conducting polymers. This was the first time that metallic-like conductivity had been reported for a biological material.

To further examine the metallic-like conductivity of the biofilms, we examined the conductive properties of the pili, which were hypothesized to form a conductive matrix through the biofilm. Pili preparations deposited as a network under physiologically relevant conditions were found to be highly conductive, conducting electrons over distances of greater than a centimeter. Pili conductivity could not be attributed to cytochromes associated with the pili. Denaturing the cytochromes had no impact on pili conductivity. Furthermore, the cytochromes on the pili were spaced too far apart for electron hoping/tunneling between cytochromes. The temperature dependence of conductance of the pili was consistent with metallic-like conductivity and the pili conductivity exhibited the expected response to proton doping. The highest conductivity of the pili was observed at pH 2, a pH that denatures cytochromes.

The relationship between pili abundance, biofilm conductivity, and the potential for current production in microbial fuel cells was further evaluated in studies with a diversity of *G. sulfurreducens* strains. These included strains in which the genes for one or more outer-surface *c*-type cytochromes was deleted as well as strain KN400, a strain selected for its superior capability for current production. These studies demonstrated that there was a strong, direct correlation between biofilm conductivity and current density. Higher conductivities also contributed to lower charge transfer resistances. Higher conductivities could clearly be attributed to higher pili abundances, consistent with the hypothesis that a network of conductive pili is responsible for long-range electron transport through the biofilms.

The alternative hypothesis that electron hoping/tunneling is responsible for the conductivity of *G. sulfurreducens* biofilms was also investigated in detail. Multiple lines of evidence, derived from studies, which relied on different basic assumptions, refuted the cytochrome hypothesis. For example, measurements of the heme content of biofilms demonstrated that there were not enough cytochromes present to account for electron conduction through the biofilms. Furthermore, there was no correlation between biofilm conductivity and cytochrome content. Biofilm conductivity did not show a redox peak, as would be expected for conduction via redox carriers, and the hoping/tunneling hypothesis was inconsistent with the observed temperature dependence of conductivity.

The mechanism for metallic-like conductivity in pili was further examined. X-ray diffraction analysis of pili preparations demonstrated the presence of overlapping pi-pi orbitals, consistent with the known role of aromatic moieties in metallic-like conductivity of synthetic organic materials. It was hypothesized that the aromatic moieties conferring metallic-like conductivity to the pili were aromatic amino acids, localized in the carboxyl terminus of PilA, the structural pilin protein. This was because the N-terminus of the PilA of *G. sulfurreducens* and microbes that produce non-conductive pili are highly conserved, but the carboxyl terminus of the *G. sulfurreducens* PilA is truncated. (b) (4)

These results definitively demonstrate that it is the intrinsic conductivity of the pili that is important for long-range electron transport along pili and through biofilms.

Studies involving electrochemical impedance spectroscopy, cyclic voltammetry and charge-discharge cycling demonstrated that *G. sulfurreducens* biofilms function as supercapacitors. The biofilms also have low self-discharge and good charge/discharge reversibility. The superior electrochemical performance of the biofilm could be related to its high abundance of cytochromes, providing large electron storage capacity, its nanostructured network with metallic-like conductivity, and its porous architecture with hydrous nature, offering prospects for future low cost and environmentally sustainable energy storage devices.

Progress was also made in understanding electron transfer from the conductive biofilm to the anode surface. Our previous studies demonstrated that the outer-surface *c*-type cytochrome OmcZ is essential for optimal current production in *G. sulfurreducens* fuel cells. In order to understand the role of OmcZ, its localization in anode biofilms was determined with immunogold/transmission electron microscopy techniques. OmcZ was highly concentrated at the biofilm-anode interface. Studies in which the potential of the anode was varied demonstrated that this was not an electrophoresis-like phenomenon. In contrast, similar studies with another outer-surface cytochrome, OmcS, revealed that this cytochrome was dispersed throughout the anode biofilm, indicating that localization at the anode surface is specific to OmcZ. These results suggest that pili conduct electrons through the biofilm and that OmcZ is required to facilitate electron transfer from the conductive biofilm to the anode surface.

The contribution of cells in different locations in the anode biofilm to current production was investigated with a novel gene reporter system employing short half-like fluorescent protein reporters. Proof of concept studies with a reporter for *niD*, which encodes one of the genes for nitrogen fixation, demonstrated that the reporter could readily detect expression of *nifD* when ammonium was excluded from the medium. Repression of *nifD* expression following the addition of ammonium was readily apparent from the loss of reporter signal. In a similar manner, the gene for the key TCA cycle enzyme, citrate synthase, was highly expressed when acetate was supplied and current levels were high. However, expression was repressed when the anode biofilms were deprived of acetate. Monitoring the expression of several metabolic genes suggested that cells throughout the anode biofilm were metabolically active and contributing to current production. This finding lends more support to the concept of long-range electron transfer through conductive anode biofilms.

The improved understanding of the components that might be important in electron transfer through biofilms and onto electrode surfaces has made it conceivable to consider genetic engineering of biofilms with enhanced current-producing capacities. (b) (4)

(b) (4)

This production of a conductive biopolymer demonstrates how basic investigations into mechanisms for electron transfer to electrodes may lead to practical improvements for microbial fuel cells and new products.

In order to determine the environmental relevance of the conductivity observed in *G. sulfurreducens* biofilms, the conductivity of 'natural' biofilms that grew on electrodes from an environmental inoculum was measured. Biofilm conductivities were high despite the fact that more than 50% of the microorganisms in the biofilm were not *Geobacter* species. These results demonstrate the significance of conductive biofilms in harvesting electricity from environmental sources.

In summary, the discovery, made under this grant, that the pili of *Geobacter sulfurreducens* are capable of long-range electron transport via metallic-like conductivity is a paradigm shift in biology. Elucidating the mechanisms for metallic-like conductivity is likely to be the key to developing strategies for improved power output in microbial fuel cells; aid in the improvement of other microbe-electrode technologies, such as electrosynthesis; and provide basic information for a host of other bioelectronics applications.

IMPACT/APPLICATIONS/TRANSISTIONS

Our ONR-supported studies received wide-spread press attention, including being named by Time Magazine as one of the top inventions for 2009. (b) (4)

REFERED PUBLICATIONS

1. Franks, A. E., K. P. Nevin, R. H. Glaven, and D. R. Lovley. 2010. Microtoming coupled with microarray analysis to evaluate potential differences in the metabolic status of *Geobacter sulfurreducens* at different depths in anode biofilms. ISME J. 4:509-519.
2. Leang, C., X. Qian, T. Mester, and D. R. Lovley. 2010. Alignment of the *c*-type cytochrome OmcS along pili of *Geobacter sulfurreducens*. Appl Environ Microbiol 76:4080-4084.
3. Inoue, K., C. Leang, A. E. Franks, T. L. Woodard, K. P. Nevin, and D. R. Lovley. 2010. Specific localization of the *c*-type cytochrome OmcZ at the anode surface in current-producing biofilms of *Geobacter sulfurreducens*. Environ. Microbiol. Rep. 3:211-217.

4. Inoue, K., X. Qian, L. Morgado, B.-C. Kim, T. Mester, M. Izallalen, C. A. Salgueiro, and D. R. Lovley. 2010. Purification and characterization of OmcZ an outer-surface, octaheme, *c*-type cytochrome essential for optimal current production by *Geobacter sulfurreducens*. Appl Environ Microbiol 76:3999-4007.
5. Klimes, A., R. H. Glaven, H. T. Tran, and D. R. Lovley. 2010. Production of pilus-like filaments in *Geobacter sulfurreducens* in the absence of the type IV PilA protein PIlA. FEMS Microb Lett 310:62-68.
6. Malvankar, N., M. Vargas, K. P. Nevin, A. E. Franks, C. Leang, B.-C. Kim, K. Inoue, T. Mester, S. F. Covalla, J. P. Johnson, V. M. Rotello, M. T. Tuominen, and D. R. Lovley. 2011. Tunable metallic-like conductivity in nanostructured biofilms comprised of microbial nanowires. Nature Nanotechnology 6:573-579.
7. Strycharz, S. M., R. H. Glaven, M. V. Coppi, S. M. Gannon, L. A. Perpetua, A. Liu, K. P. Nevin, and D. R. Lovley. 2011. Gene expression and deletion analysis of mechanisms for electron transfer from electrodes to *Geobacter sulfurreducens*. Bioelectrochemistry 80:142-150.
8. Strycharz, S. M., A. P. Malanoski, R. M. Snider, H. Yi, D. R. Lovley, and L. M. Tender. 2011. Application of cyclic voltammetry to investigate enhanced catalytic current generation by biofiolm-modified anodes of *Geobacter sulfurreducens* strain DL1 vs variant strain KN400. Energy & Environ. Sci. 4:896-913.
9. Lovley, D. R., and K. P. Nevin. 2011. A shift in the current: new applications and concepts for microbe-electrode electron exchange. Curr Opin Biotechnol 22:441-448.
10. Lovley, D. R. 2011. Live wires: direct extracellular electron exchange for bioenergy and the bioremediation of energy-related contamination. Energy & Environmental Science 4:4896-4906.
11. Lovley, D. R., T. Ueki, T. Zhang, N. S. Malvankar, P. M. Shrestha, K. Flanagan, M. Aklujkar, J. E. Butler, L. Giloteaux, A.-E. Rotaru, D. E. Holmes, A. E. Franks, R. Orellana, C. Risso, and K. P. Nevin. 2011. Geobacter: the microbe electric's physiology, ecology, and practical applications. Adv. Microb. Physiol. 59:1-100.
12. Franks, A. E., R. H. Glaven, and D. R. Lovley. 2012. Real-time spatial gene expression analysis within current-producing biofilms. ChemSusChem 5:1092-1098.
13. Lovley, D. R. 2012. Electromicrobiology. Ann. Rev. Microbol. 66:391-409.
14. Malvankar, N. S., J. Lau, K. P. Nevin, A. E. Franks, M. T. Tuominen, and D. R. Lovley. 2012. Electrical conductivity in a mixed-species biofilm. Appl. Environ. Microbiol. 78:5967–5971.
15. Malvankar, N. S., and D. R. Lovley. 2012. Microbial nanowires: a new paradigm for biological electron transfer and bioelectronics. ChemSusChem 5:1039– 1046.
16. Malvankar, N. S., T. Mester, M. T. Tuominen, and D. R. Lovley. 2012. Supercapcitors based on c-type cytochromes using conductive nanostructured networks of living bacteria. ChemPhysChem 13:463-468.
17. Malvankar, N. S., M. T. Tuominen, and D. R. Lovley. 2012. Biofilm conductivity as a decisive variable for the high-current-density Geobacter sulfurreducens microbial fuel cells. Energy & Environ. Sci. 5:5790-5797.
18. Malvankar, N. S., M. T. Tuominen, and D. R. Lovley. 2012. Biofilm conductivity is a decisive variable for high-current-denisty Geobacter sulfurreducens microbial fuel cells. Energy. Environ. Sci. 5:5790-5797.
19. Malvankar, N. S., M. T. Tuominen, and D. R. Lovley. 2012. Comment on "On electrical conductivity of microbial nanowires and biofilms". Energy. Environ. Sci. 5:6247-6249.
20. Malvankar, N. S., M. T. Tuominen, and D. R. Lovley. 2012. Lack of invovlement of c-type cytochromes in long-range electron transport in microbial biofilms and nanowires. Energy. Environ. Sci. 5: 8651 - 8659.

21. Vargas, M., N. S. Malvankar, P.-L. Tremblay, C. Leang, J. A. Smith, P. Patel, O. Synoeyenbos-West, K. P. Nevin, and D. R. Lovley. 2013. Aromatic amino acids required for pili conductivity and long-range extracellular electron transport in Geobacter sulfurreducens mBio **4:**4:e00105-13.